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Isolation of Citrate-Positive Variants of *Escherichia coli* from Domestic Pigeons, Pigs, Cattle, and Horses

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Twenty-seven isolates of citrate-positive variants of *Escherichia coli* were obtained from domestic pigeons, pigs, cattle, and horses. With the exception of citrate utilization, all isolates closely resembled typical *E. coli* in their biochemical reactions. These isolates were multiply resistant to antibiotics in in vitro susceptibility tests. Transfer experiments of multiple-drug resistance to the *E. coli* K-12 strain showed that all citrate-positive isolates from domestic pigeons, pigs, and cattle, resistant to three or more drugs, carried R plasmids showing temperature-sensitive transfer.

The utilization of citrate on Simmons citrate agar, to which sodium citrate was added as a source of carbon and energy, is very important and valuable for identification in the family *Enterobacteriaceae*. It has been accepted that most of the species of the tribes *Salmonelleae*, *Klebsielleae*, and *Proteeae* possess the citrate-utilizing ability to grow on the medium, but other tribes, e.g., *Escherichieae* and *Edwardsielleae*, do not possess it.

The incidence of citrate-positive *Escherichia coli* variants has been very low. Edwards and Ewing reported (2) that 0.6% of cultures taken at random from materials submitted for identification and 0.9% of 210 standard O-, K-, and H-antigen strains were positive on Simmons citrate agar. Recently, Washington and Timm (10) reported that three strains isolated from cultures of clinical materials from human sources were typical *E. coli* in their biochemical reactivity, with the exception of their ability to grow on the Simmons citrate agar. However, there has been no report of isolation of the variants from non-human sources.

Recently, we detected a high prevalence of *E. coli*-like variants among isolates from domestic pigeons, pigs, cattle, and horses. This paper deals with the isolation of the variants, their biochemical reactivity and antibiotic susceptibility, and detection of conjugative R plasmids.

MATERIALS AND METHODS

Procedure for isolation of *E. coli*-like colonies from fecal samples. (i) Domestic pigeons. Cloacal swab samples were obtained from 22 domestic pigeons. The swabs were incubated in 10 ml of Selenite brilliant green broth (Eiken) at 43°C overnight. Then the broth cultures were subcultured onto brilliant green agar plates (Eiken) to isolate *Salmonella*. These broth cultures were left in an incubator at 37°C overnight to enhance *E. coli* growth and then were subcultured onto MacConkey-lactose agar (Eiken) plates. One or two *E. coli*-like colonies were picked up from each sample, and a total of 36 isolates were used in this study.

(ii) Pigs. The composite fecal samples were collected from the floor of 33 pens of two pig houses. Thirty-three *E. coli*-like colonies were obtained by direct cultivation, using MacConkey agar plates.

(iii) Cattle. Fecal samples were collected from seven cattle on a farm. After these samples were put into Selenite brilliant green broth and incubated at 43°C overnight for *Salmonella* isolation, these broth cultures were left at 37°C overnight and were subcultured onto deoxycholate-hydrogen sulfide-lactose agar (DHL; Eiken) plates with or without the addition of chloramphenicol (CP; 25 μg/ml) to select drug-resistant *E. coli*.

(iv) Horses. Rectal fecal samples were obtained from 10 horses on a farm. Approximately 1 g of these rectal feces was suspended in 1 ml of saline, and 1 loopful of the saline suspension was plated on DHL agar plates with or without the following antibiotics: CP, 25 μg/ml; tetracycline (TC), 25 μg/ml; streptomycine (SM), 12.5 μg/ml.

Test for growth on Simmons citrate agar. The inoculations were always conducted by streaking the saline bacterial suspension onto Simmons citrate agar slants or plates. A positive reaction of citrate medium was demonstrated by both the alkalization of the citrate medium and the presence of colonies. Also, the effect of various incubation temperatures (22, 37, and 43°C) on growth of citrate-positive, *E. coli*-like isolates on the Simmons citrate agar slants was examined.

Differential tests of *E. coli*. Preliminary identification of isolates from the samples was determined by an initial set of tests that are used routinely in this laboratory for identification of gram-negative bacilli: triple sugar iron agar, sulfide-indole-motility medium, Simmons citrate agar, Voges-Proskauer reaction, and lysine-iron agar.

Citrate-positive and -negative *E. coli*-like isolates

were identified by biochemical tests described by Edwards and Ewing (2). A neotype *E. coli* strain (ATCC 11775) was used as a control. No serological studies were performed.

Antibiotic susceptibility tests. Antibiotic susceptibility testing of the isolates was performed by the agar dilution method with a multiple inoculator, using 12 antibiotics at the following final concentrations (micrograms per milliliter): CP, 25; TC, 25; SM, 12.5; kanamycin, 25; aminobenzylpenicillin, 25; cephaloridine, 25; gentamicin, 12.5; colistin, 12.5; furatrizine, 6.3; nalidixic acid, 25; rifampin, 25; and sulfadimethoxine (SA), 800. Heart infusion agar (Eiken) was used for the test, except in the tests with SA, in which Mueller-Hinton agar (Eiken) was used. A loopful of an approximately 10^{-3} dilution of a overnight broth culture was inoculated onto the media which contained antibiotics. An isolate was recorded as resistant if its growth was not inhibited by these concentrations of the drugs.

Detection of conjugative R plasmids. The detection of R plasmids was made according to the procedures noted by Sato et al. (5, 6). *E. coli* ML1410 (nalidixic acid-resistant, methionine-requiring F^- derivative of K-12) was used as a recipient. Each of the isolates was cultivated in brain heart infusion broth (Eiken) at 37°C for 18 h. *E. coli* ML1410 was cultured in a similar way. Two milliliters of broth in a test tube was inoculated with 0.2 ml of each donor broth culture and an equal amount of recipient culture. The mixture was incubated at 37°C for 18 h. A loopful of each mixed culture was subcultured onto a selective agar plate containing nalidixic acid (50 μg/ml) and one of the following drugs to which the isolate tested was resistant: CP (25 μg/ml), SM (12.5 μg/ml), TC (25 μg/ml), kanamycin (25 μg/ml), aminobenzylpenicillin (25 μg/ml), or SA (800 μg/ml). In this experiment, a heart infusion agar was used for TC, Mueller-Hinton agar was used for SA, and DHL agar was used for CP, SM, kanamycin, and aminobenzylpenicillin. To the heart infusion or Mueller-Hinton agar were added 4 ml of a 0.2% BTB solution and 1.5 g of lactose per 100 ml. The subcultured selective media were incubated overnight at 37°C. The transferred resistance marker was determined by observing colonies grown on each selective medium. To determine transconjugant recipients and their resistance patterns, three colonies of transconjugants on each selective medium were purified and examined for resistance to the antibiotics applied. When the transconjugant recipient was not found on the selective media, the mixed cultures, which had been left overnight at room temperature (about 25°C), were reinoculated on the selective media to detect temperature-sensitive R plasmids (7). If the transconjugant was not detected by the mating culture made at 37°C, but rather was detected only by the mating culture at room temperature, or if the transconjugant was more dominantly detected by the mating culture made at 25°C than that made at 37°C, donor cultures were tested further to confirm whether the R plasmids were temperature sensitive or not. Transfer frequency of the R plasmids was estimated by using mating cultures made at 25 or 37°C (7).

RESULTS

Isolation of citrate-positive *E. coli*-like colonies. A total of 36 *E. coli*-like isolates were obtained from 22 domestic pigeons (Table 1). Thirty-three *E. coli*-like colonies were obtained from 33 composite porcine fecal samples. Of 24 *E. coli*-like isolates from seven cattle, three isolates from three cattle were picked up on DHL agar containing CP. Fourteen isolates obtained from 10 horses contained one CP-resistant culture, one TC-resistant culture, and three SM-resistant cultures, which were obtained on each selective medium containing CP, TC, and SM, respectively. Among the *E. coli*-like isolates from domestic pigeons, pigs, cattle, and horses, 13 (36.1%), 12 (36.3%), 1 (4.1%), and 1 (7.1%), respectively, were found to be citrate positive (Table 1).

Tests on Simmons citrate agar. Most of the citrate-positive isolates alkalized Simmons citrate agar within 2 days of incubation at 37°C. Colonies ranged in size from 0.6 to 1.8 mm on Simmons citrate agar plates after 4 days of incubation at 37°C. When several colonies of some isolates grown on DHL agar plates were inoculated onto Simmons citrate agar, occasionally no growth on the media was observed. This seemed to indicate instability of citrate utilization by *E. coli* isolates. All 27 *E. coli*-like isolates from domestic pigeons, pigs, cattle, and horses were tested repeatedly on different lots of Simmons citrate agar produced by three different manufacturers: Difco (United States), Eiken (Japan),

TABLE 1. *Isolation of citrate-utilizing E. coli variants from domestic pigeons, pigs, cattle, and horses in the Hokkaido district*

Host	Locality	Period of isolation	No. of locations	No. of birds or animals tested	Specimens examined	No. of *E. coli*	No. of isolates utilizing citrate (%)
Pigeons	Sapporo	28 Nov. 1974–15 Jan. 1975	1	22	Cloacal swabs	36	13 (36.1)
Pigs	Chitose	30 Jun. 1973	1	33	Composite fecal samples	33	12 (36.3)
Cattle	Soya	19 Jun. 1975	1	7	Rectal feces	24 (3)[a]	1 (4.1)
Horses	Kushiro	22–23 Nov. 1976	1	10	Rectal feces	14 (5)[a]	1 (7.1)

[a] Number in parentheses is number of isolates obtained on selective media containing antibiotics.

and Nissan (Japan). Consequently, the results on each of the different lots within 4 days at 37°C were consistently positive (Table 3). The effect of various incubation temperatures on the growth of citrate-positive isolates is shown in Table 2. When these citrate-positive isolates were incubated at 22 or 37°C for 4 days, all isolates were positive on Simmons citrate agar slants. However, when the same isolates were incubated at 43°C for 4 days, five (38.4%) isolates from pigeons and two (16.6%) isolates from pigs utilized citrate, but growth of the isolates was delayed at this incubation temperature. When the slants incubated at 43°C for 4 days were separately incubated at 43 or 37°C for 4 more days, the slants replaced to 37°C changed into a positive reaction, but those left at 43°C did not. An incubation temperature of 43°C evidently hindered citrate utilization by the *E. coli* isolates.

Biochemical characteristics. According to the initial tests used, with the exception of alkalization of Simmons citrate agar, all 107 *E. coli*-like isolates from domestic pigeons, pigs, cattle, and horses closely resembled *E. coli*. All of the citrate-positive and citrate-negative isolates were identified as *E. coli* by biochemical tests described by Edwards and Ewing and as shown in Table 3. The high frequency of positive reactions in xylose and raffinose was recorded, but the findings were not considered specific to nonhuman isolates of *E. coli* on the basis of extensive surveys in previous reports. Generally speaking, with the exception of the citrate reaction, all the citrate-positive isolates could not be distinguished from the citrate-negative isolates and the neotype *E. coli* strain ATCC 11775.

Antibiotic susceptibility and detection of conjugative R plasmids. The antibiotic susceptibility of all isolates tested and resistance patterns of their conjugative R plasmids are listed in Table 4. All of the isolates were susceptible to gentamicin, colistin, cephaloridine, nalidixic acid, and rifampin. All of the citrate-positive variants from pigeons, pigs, and a cow were resistant to three or more drugs such as: TC, CP, SM, SA, kanamycin, and aminobenzylpenicillin. These variants had R plasmids conferring multiple resistance (Table 4). In particular, the resistance pattern of R plasmids of all of the 13 variants of pigeons was TC-CP-SM-SA.

Citrate-negative isolates from pigeons and pigs which indicated multiple resistance did not always contain R plasmids. The citrate-positive variants from cattle had an R plasmid conferring resistance to TC, CP, SM, and SA. However, a citrate-positive variant from a horse was susceptible to the 12 antibiotics tested. In particular, it was of interest that the resistance of all citrate-positive variants from pigeons, pigs, and cattle was more efficiently transferred to *E. coli* ML1410 at 25°C than at 37°C, indicating that their R plasmids were thermosensitive.

TABLE 2. *Effect of various incubation temperatures on growth of citrate-utilizing E. coli on Simmons citrate agar slants*

Host	No. of isolates	No. of isolates grown on Simmons citrate agar slants (%)				
		After incubation for 4 days at:			After reincubation for 4 more days[a] at:	
		22°C	37°C	43°C	43°C	37°C
Pigeons	13	13/13 (100)	13/13 (100)	5/13 (38.5)	5/13 (38.5)	11/13 (84.6)
Pigs	12	12/12 (100)	12/12 (100)	2/12 (16.6)	2/12 (16.6)	7/12 (58.3)
Cow	1	1	1	1		
Horse	1	1	1	1		

[a] Duplicates of Simmons citrate agar slants were inoculated and incubated at 43°C for 4 days. Then they were separately incubated at 43 and 37°C for 4 more days.

DISCUSSION

The incidence of citrate-positive *E. coli* variants is reported to be very rare (2, 10). In the present study, citrate-positive *E. coli* variants were isolated from pigeons, pigs, a cow, and a horse with high frequency. Apart from their ability to utilize citrate on Simmons citrate agar, these citrate-positive *E. coli* variants, as well as citrate-negative isolates, agreed with the pattern of the neotype *E. coli* strain ATCC 11775 in their biochemical reaction. Their ability to utilize citrate within 4 days was reproducible on different lots of agar at temperatures below 37°C, whereas most of the isolates from pigeons and pigs were inhibited in growing on the citrate medium at 43°C for 8 days (Table 2). All the 27 citrate-positive *E. coli* variants except that isolated from a horse were resistant to three to six drugs and were found to carry conjugative R plasmids (Table 4). Moreover, it was of interest that multiple-drug resistance of all of the 26 citrate-positive *E. coli* variants isolated from pigeons, pigs, and cattle was transferred more efficiently at 25 than at 37°C.

Further studies indicate that transfer of the R plasmids is temperature sensitive but their replication is not (8). In addition, the plasmids belonged to incompatibility group H (9). As mentioned above, there seemed to be a tendency for spontaneous loss of the citrate-utilizing character in a part of the isolates, especially with incubation at 43°C, and most of the citrate-positive isolates carried conjugative R plasmids. These findings suggest that the citrate-utilizing character observed in this study may be me-

TABLE 3. *Thirty-four biochemical reactions of 27 citrate-positive (Cit$^+$) and 80 citrate-negative (Cit$^-$) E. coli isolates from domestic pigeons, pigs, cattle, and horses*

Test or substrate	Pigeons (36)[a]				Pigs (33)				Cattle (24)			Horses (14)			Neotype *E. coli* strain ATCC 11775
	Cit$^+$ (13)[a]		Cit$^-$ (23)[a]		Cit$^+$ (12)		Cit$^-$ (21)		Cit$^+$ (1)	Cit$^-$ (23)		Cit$^+$ (1)	Cit$^-$ (13)		
	No.	%	No.	%	No.	%	No.	%		No.	%		No.	%	
Citrate (Simmons)															
Eiken	13	100	0	0	12	100	0	0	+	0	0	+	0	0	−
Nissan	13	100	0	0	12	100	0	0	+	0	0	+	0	0	−
Difco	13	100	0	0	12	100	0	0	+	0	0	+	0	0	−
ONPG[b]	13	100	23	100	12	100	21	100	+	23	100	+	13	100	+
Hydrogen sulfide	0	0	0	0	0	0	1	4.7	−	0	0	−	3	23.0	−
Urease (Christensen)	0	0	0	0	0	0	0	0	−	0	0	−	0	0	−
Indole	13	100	23	100	12	100	21	100	+	23	100	+	13	100	+
Methyl red	13	100	23	100	12	100	21	100	+	23	100	+	13	100	+
Voges-Proskauer	0	0	0	0	0	0	0	0	−	0	0	−	0	0	−
KCN	0	0	0	0	0	0	0	00	−	0	0	−	0	0	−
Motility	13	100	21	91.3	4	33.3	6	28.5	+	21	91.3	+	12	92.3	+
Gelatin, 22°C	0	0	0	0	0	0	0	0	−	0	0	−	0	0	−
Lysine decarboxylase	13	100	23	100	12	100	21	100	+	23	100	+	13	100	+
Arginine dihydrolase	13	100	2	8.7	3	25.0	0	0	−	2	8.7	−	3	23.0	−
Ornithine decarboxylase	13	100	20	86.9	7	58.3	14	66.6	+	23	100	+	12	92.3	+
Phenylalanine deaminase	0	0	0	0	0	0	0	0	−	0	0	−	0	0	−
Malonate	0	0	0	0	0	0	0	0	−	0	0	−	0	0	−
Glucose	13	100	23	100	12	100	21	100	+	23	100	+	13	100	+
Lactose	13	100	23	100	12	100	21	100	+	23	100	+	13	100	+
Sucrose	0	0	20	86.9	2	16.6	8	38.1	+	2	8.7	+	12	92.3	−
Mannitol	13	100	23	100	12	100	20	95.2	+	23	100	+	12	92.3	+
Dulcitol	13	100	22	95.6	6	50.0	14	66.6	+	21	91.3	+	10	76.9	−
Salicin	3	23.0	19	82.6	10	83.3	15	71.4	+	20	86.9	+	12	92.3	−
Adonitol	0	0	1	4.3	1	8.3	2	9.5	−	0	0	−	0	0	−
Inositol	0	0	0	0	0	0	0	0	−	1	4.3	−	1	7.6	−
Sorbitol	13	100	23	100	11	91.6	19	90.4	+	23	100	+	13	100	+
Arabinose	13	100	23	100	12	100	21	100	+	23	100	+	13	100	+
Raffinose	13	100	23	100	4	33.3	7	33.3	−	22	95.6	+	12	92.3	−
Rhamnose	13	100	23	100	11	91.6	21	100	+	23	100	+	12	92.3	+
Maltose	13	100	23	100	12	100	21	100	+	23	100	+	13	100	+
Xylose	13	100	23	100	12	100	21	100	+	23	100	+	13	100	−
Trehalose	13	100	23	100	12	100	21	100	+	23	100	+	13	100	+
Nitrate to nitrite	13	100	23	100	12	100	21	100	+	23	100	+	13	100	+
Sodium acetate	13	100	23	100	12	100	20	95.2	+	23	100	+	11	84.6	+
Oxidase	0	0	0	0	0	0	0	0	−	0	0	−	0	0	−
Esculin	0	0	13	56.5	8	66.6	11	52.3	+	16	69.5	−	5	38.4	−

[a] Total number of isolates.
[b] ONPG, *o*-Nitrophenyl-β-D-galactopyranoside.

diated by plasmids, as has been shown in H_2S production in *E. coli* (1, 3, 4). Futher studies on this problem showed that the citrate-utilizing ability in such *E. coli* variants of pigeon, pig, and bovine origin is plasmid mediated (Sato et al., The 2nd Tokyo Symposium on Microbial Drug Resistance, 26–28 October, 1977).

The citrate reaction on Simmons citrate agar was considered a key characteristic for identification of *Enterobacteriaceae* as well as H_2S production. However, there has been no information on the degree of relative frequency of citrate-positive *E. coli* variants in nature, especially in nonhuman sources.

Extensive information needs to be gathered concerning the distribution of citrate-positive *E. coli* variants in humans and in various other animals.

TABLE 4. *Drug resistance patterns and conjugative R plasmids in citrate-positive and -negative E. coli isolates*[a]

Host	No. of isolates	Citrate utilization (no. of isolates)	Drug resistance		Resistance patterns of conjugative R plasmids	No. of R^+ isolates	No. of ts[b] R^+ isolates
			Resistance patterns	No. of isolates			
Pigeons	36	+(13)	TC-CP-SM-SA	13	TC-CP-SM-SA	13	13
		−(23)	TC-CP-SM-SA	15	TC-CP-SM-SA	15	0
			TC-SM-SA	2	TC-SM-SA	2	0
			SA	2	—[c]		
			(s)[d]	4			
Pigs	33	+(12)	TC-CP-SM-SA-KM-APC	1	TC-CP-SA-KM	1	1
			TC-CP-SM-SA-KM	6	TC-CP-SM-SA-KM	6	6
			TC-CP-SM-SA	1	TC-CP-SM-SA	1	1
			TC-CP-SM-SA-FT	3	TC-CP-SM-SA	3	3
			TC-CP-SA	1	TC-CP-SA	1	1
		−(21)	TC-CP-SM-SA-KM-APC	1	TC-CP-KM-APC	1	0
			TC-CP-SM-SA-KM	1	TC-CP-SM-KM	1	0
			TC-CP-SA-KM-APC	1	TC-CP-KM-APC	1	0
			TC-CP-SM-SA	3	TC-CP-SM-SA	2	2
			TC-CP-SM-SA-FT	1	SM-SA	1	0
			CP-SM-SA-KM-APC	1	CP-SM-SA-APC	1	0
			CP-SA-FT	1	CP-SA	1	0
			CP-KM-APC-FT	1	CP-APC	1	0
			TC-SM-SA-KM-FT	2	SM-SA-KM	2	0
			TC-SM-SA-KM	1	—		
			TC-SM-SA-FT	1	SM-SA	1	0
			TC-SM-KM-FT	3	TC-SM-KM	1	0
			TC-SM-SA	1	—		
			TC-SA	1	—		
			KM	1	—		
			(s)	1			
Cattle	27	+(1)	TC-CP-SM-SA	1	TC-CP-SM-SA	1	1
		−(26)	TC-CP-SM-SA	3	TC-SM-SA	1	0
					TC	1	0
			TC-SM-SA	3	TC-SM-SA	2	0
			TC-SM-KM	2	TC-SM-KM	1	0
					SM-KM	1	0
			TC-CP-SA	1	—		
			TC	1	—		
			SM	2	SM	1	0
			(s)	14			
Horses	14	+(1)	(s)	1			
		−(13)	TC-SM-KM-APC	1	TC-SM-KM-APC	1	0
			CP-SM-SA-APC	1	CP-SM-SA-APC	1	0
			SM-SA	2	—		
			SM	5	SM	1	0
			(s)	4			

[a] Abbreviations: TC, Tetracycline; SM, streptomycin; CP, chloramphenicol; SA, sulfadimethoxine; KM, kanamycin; APC, aminobenzylpenicilin; FT, furatrizine.

[b] ts, R plasmids showing temperature-sensitive transfer (7).

[c] —, Resistance not transferred.

[d] (s), Susceptibility to antibiotics used in the present study.

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